Variants of acetylserotonin o-methyltransferase and uses thereof

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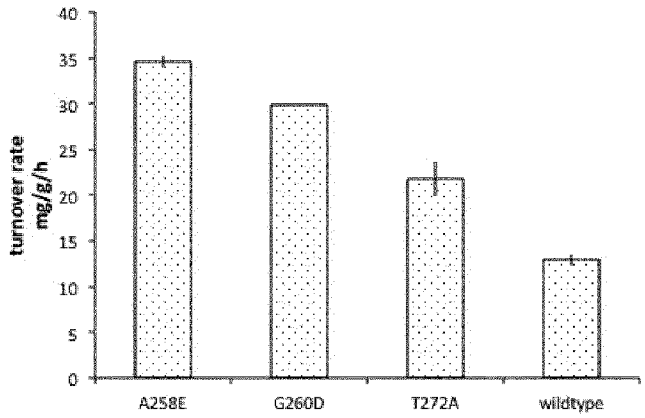
FIG. 2



(57) Abstract: Described herein are variants of acetylserotonin O-methyltransferase (ASMT) as well as vectors and recombinant microbial host cells expressing such ASMT variant and their use in producing melatonin and related compounds. Preferred ASMT variants provide for a higher turnover of N-acetylserotonin into melatonin.

WO 2017/202897 A1

VARIANTS OF ACETYLSEROTONIN O-METHYLTRANSFERASE AND USES THEREOF

FIELD OF THE INVENTION

The present invention relates to variants of acetylserotonin O-methyltransferase (ASMT) as well as vectors and recombinant host cells expressing such ASMT variant and their use in producing melatonin. More specifically, the present invention relates to ASMT variants providing for a higher turnover of N-acetylserotonin into melatonin.

BACKGROUND OF THE INVENTION

Melatonin is a powerful antioxidant and maintains the body's circadian rhythm. Over-the-counter dietary supplements based on melatonin have been available for many years in the U.S. While commercial melatonin is typically chemically synthesized, melatonin can also be produced in microbial cells engineered to express an appropriate biosynthetic pathway (see, *e.g.*, WO 2013/127915 A1, WO 2015/032911 A1 and US 2014/134689 AA).

In animals, melatonin is biosynthesized from the native metabolite L-tryptophan via the intermediates 5-hydroxy-L-tryptophan (5HTP), serotonin and N-acetylserotonin. The last step is this pathway, the conversion of N-acetylserotonin and S-adenosyl-L-methionine (SAM) to melatonin and S-adenosyl-L-homocysteine (SAH), is catalyzed by ASMT. The SAH can then be recycled back to SAM via the S-adenosyl-L-methionine cycle in microbial cells where the S-adenosyl-L-methionine cycle is native (*e.g.*, in *E. coli*) or recombinantly introduced. Byeon *et al.* (2016) and Kang *et al.* (2011) describe *Arabidopsis* and plant ASMTs, respectively.

Botros *et al.* (2013) explored the crystal structure of human ASMT (hereinafter "*Homo sapiens* ASMT" or "hsASMT"), described as consisting of a C-terminal domain typical of other SAM-dependent O-methyltransferases, and an N-terminal domain which intertwines several helices with another monomer to form the physiologically active dimer. They also analyzed 20 nonsynonymous hsASMT variants for their activity, finding that the majority of these mutations reduced or abolished ASMT activity. For example, the naturally occurring hsASMT variants P243L, Y248H and I269M exhibited a reduced or abolished enzyme activity. In addition, based on both genetics and biochemical data, it was proposed in WO 2007/052166 (Institut Pasteur) that mutations in the ASMT gene cause an absence or a decrease of melatonin and confer an increased risk to neuropsychiatric disorders such as ASD and ADHD.

For the purpose of biosynthetic production of melatonin, however, there is a need for ASMT variants and recombinant cells providing for an improved production of melatonin.

SUMMARY OF THE INVENTION

The present inventor has found that, surprisingly, mutations in certain residues of ASMT can increase the turnover of N-acetylserotonin into melatonin. In particular, mutations located in or near the opening for the SAM-binding site are advantageous, such as mutations in or structurally adjacent to the helix "guarding" the opening. It was also found that a deletion or downregulation of a gene encoding a cyclopropane fatty acyl phospholipid synthase can increase the yield of melatonin.

Accordingly, in one aspect, the invention relates to a variant of a parent ASMT, the variant comprising a mutation in at least one residue located in the segment corresponding to residues A258-T272, P241-Y248, D259-H271 and/or T307-Q310 of hsASMT, the mutation providing for an increased catalytic activity as compared to the parent ASMT. Examples of ASMTs include, but are not limited to, those listed in Table 1. Preferred mutations include those corresponding to A258E, G260D and T272A in hsASMT.

In other aspects, the invention relates to nucleic acid sequences and vectors encoding such variant ASMTs.

The invention also relates to recombinant host cells. In one aspect, the recombinant host cell comprises such variant ASMTs, nucleic acids and/or vectors. In one aspect, the recombinant host cell is derived from an *E. coli* cell and comprising a heterologous biosynthetic pathway for producing melatonin and a deletion or downregulation of the *cfa* gene.

In other aspects, the invention relates to the use of such recombinant host cells for producing melatonin.

These and other aspects and embodiments are described in more detail below.

LEGEND TO THE FIGURES

Fig. 1: Metabolic pathways for the production of melatonin according to the invention.

Fig. 2: ASMT turnover rate measurement. Cells expressing various hsASMT variant were grown in M9 medium supplemented with 200 mg/l of acetylserotonin at 37°C and secretion of melatonin was determined at different time points.

Fig. 3: Protein sequence alignment of 6 functional ASMTs; hsASMT (SEQ ID NO:1), caffeic acid o-methyltransferase from *Ocimum basilicum (*atASMT*;* SEQ ID NO:2), bovine (*Bos*

Taurus) ASMT (btASMT; SEQ ID NO:3), *Takifugu rubripes* ASMT (trASMT; SEQ ID NO:4), *Macaca mulatta* ASMT (mamuASMT; SEQ ID NO:5) and *Elephantulus edwardii* ASMT (eeASMT; SEQ ID NO:6). All of these ASMTs have been functionally tested in *E.coli*.

DETAILED DISCLOSURE OF THE INVENTION

As shown in Example 1 and in Figure 1, surprisingly, it is possible to improve the catalytic activity of ASMT by engineering specific residues in or near the opening for the SAM-binding site.

In one aspect, the variant ASMT comprises or consists of a mutation in at least one of the residues corresponding to A258, G260 and T272 in *Homo sapiens* ASMT (hsASMT; SEQ ID NO:1). In separate and specific embodiments, the variant ASMT is a variant of hsASMT (SEQ ID NO:1), atASMT (SEQ ID NO:2), btASMT (SEQ ID NO:3), trASMT (SEQ ID NO:4), mamuASMT (SEQ ID NO:5) or eeASMT (SEQ ID NO:6), such as, *e.g.*, hsASMT (SEQ ID NO:1).

In one aspect, the variant ASMT is a variant of *Homo sapiens* ASMT (SEQ ID NO:1), comprising at least one mutation in the segment corresponding to residues A258 to T272, the mutation providing for an increased catalytic activity in converting N-acetylserotonin to melatonin. In one embodiment, the variant comprises a mutation in a residue selected from the group consisting of A258, G260, and T272.

In one aspect, the variant ASMT is a variant of *Homo sapiens* ASMT, the variant having at least 92% sequence identity to *Homo sapiens* ASMT (SEQ ID NO:1) and comprising one or more mutations in the segment corresponding to residues A258 to T272 in *Homo sapiens* ASMT (SEQ ID NO:1), providing for an increased catalytic activity in converting N-acetylserotonin to melatonin as compared to hsASMT. The variant ASMT, may, for example, have at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% sequence identity to *Homo sapiens* ASMT (SEQ ID NO:1). In one embodiment, the variant comprises a mutation in a residue selected from the group consisting of A258, G260, and T272.

In specific embodiments, the variant ASMT may have at least about 50%, such as at least about 100%, such as at least about 150%, such as at least about 200% increased catalytic activity in converting N-acetylserotonin to melatonin as compared to hsASMT.

In some embodiments, the mutation in the variant ASMT is an amino acid substitution selected from the group consisting of (a) A258E; (b) G260D, G260N, G260L, and G260I; and (c) T272A and T272G. In a specific embodiment, the mutation is A258E.

In one aspect, there is provided a variant ASMT comprising a sequence selected from SEQ ID NO:1-6, or a catalytically active fragment of any thereof, having a A258E, G260D, G260N, G260L, G260I, T272A or T272G mutation, such as a A258E, G260D or T272A mutation.

In one aspect, there is provided a variant ASMT comprising SEQ ID NO:1, or a catalytically active fragment thereof, having a A258E, G260D, G260N, G260L, G260I, T272A or T272G mutation, such as a A258E, G260D or T272A mutation.

In another aspect, there is provided a nucleic acid sequence encoding the variant ASMT of any one of the aspects or embodiments described herein. The nucleic acid sequence may be comprised in a vector, and optionally operably linked to one or more expression control sequences.

In another aspect, there is provided a recombinant host cell comprising any such variant ASMT, nucleic acid sequence or vector. The recombinant host cell can, for example, be derived from a mammalian cell, a bacterial cell, a yeast cell, a filamentous fungal cell or an algal cell. In one embodiment, the recombinant host cell is derived from an Escherichia cell. The recombinant host cell may further comprise a native or heterologous biosynthetic pathway for producing N-acetylserotonin and a native or heterologous pathway for recycling S-adenosyl-L-homocysteine (SAH) into S-adenosyl-L-methionine (SAM). Optionally, the recombinant host cell may also comprise a deletion or downregulation of a gene encoding a cyclopropane fatty acyl phospholipid synthase.

In another aspect, there is provided a recombinant host cell derived from an *E. coli* cell, comprising an ASMT, a native or heterologous biosynthetic pathway for producing N-acetylserotonin, a native or heterologous pathway for recycling SAH into SAM, and a deletion or downregulation of the *cfa* gene.

In another aspect, there is provided a method of producing melatonin, comprising culturing the recombinant host cell of any aspect or embodiment described herein in a medium comprising a carbon source, and, optionally, isolating melatonin.

Definitions

Unless otherwise specified or contradicted by context, "*Homo sapiens* ASMT", "human ASMT" or "hsASMT" herein refers to the sequence provided by NCBI GI# 1170276 and Uniprot reference P46597-1 (SEQ ID NO:1). ASMT is also known as hydroxyindole O-methyltransferase (HIOMT).

As used herein, "exogenous" means that the referenced item, such as a molecule, activity or pathway, is added to or introduced into the host cell or microorganism. An exogenous nucleic acid sequence can, for example, be introduced either as chromosomal genetic material by integration into a host chromosome or as non-chromosomal genetic material such as a plasmid. Such an exogenous nucleic acid sequence can encode an enzyme or enzyme activity which is either heterologous to the host cell or organism in question or which is an endogenous enzyme or enzyme activity in the host cell or organism. Likewise, an exogenous molecule such as a substrate or cofactor can be added to or introduced into the host cell or microorganism, *e.g.*, via adding the molecule to the media in or on which the host cell or microorganism resides.

In the present context the term "heterologous" means that the referenced item, such as a molecule, activity or pathway, does not normally appear in the host cell or microorganism species in question. Typically, a heterologous pathway comprises at least one enzyme or other component which is heterologous to the host cell.

As used herein, the terms "native" or "endogenous" mean that the referenced item is normally present in or native to the host cell or microbial species in question.

As used herein, "upregulating" an endogenous gene means increasing the transcription and/or translation of a gene present in the native host cell genome relative to a control, such as *e.g.* the unmodified host cell. Methods of upregulating genes are known in the art and include, *e.g.*, introducing a non-native promoter increasing transcription, modifying the native promoter, deleting genes encoding repressor protein, introducing multiple copies of the gene of interest, etc. "Downregulating" an endogenous gene as used herein means to reduce, optionally eliminate, the transcription or translation of an endogenous gene relative to a control, such as, *e.g.*, the unmodified host cell. Methods of down-regulating, disrupting and deleting genes are known to those of skill in the art, and include, *e.g.*, site-directed mutagenesis, genomic modifications based on homologous recombination, RNA degradation based on CAS9, etc.

In the present context, "overexpressing" refers to introducing an exogenous nucleic acid sequence encoding an enzyme which is either heterologous or native to the microbial host cell, or is a functionally active fragment or variant thereof, and expressing the exogenous nucleic acid sequence to increase the enzyme activity in the microbial cell as compared to the microbial host cell without the introduced exogenous nucleic acid sequence, *e.g.*, a native microbial host cell. This can be useful if, *e.g.*, a microbial host cell does not normally contain the enzymatic activity referred to, where the native enzymatic activity is insufficient, or the native enzyme is subjected to unwanted regulation. In such cases, an exogenous nucleic acid sequence encoding an enzyme which is heterologous to the microbial host cell and which has the desired activity and regulation patterns can be introduced. Overexpression of a nucleic acid sequence can be achieved by placing the nucleic acid sequence under the control of a strong promoter. Non-limiting examples of strong promoters suitable for, *e.g.*, *E. coli* cells are Ptrc, Plac, PlacUV5, PT7, and PTrp. Non-limiting examples of strong promoters suitable for, *e.g.*, yeast cells are TEF1, PGK1, HXT7 and TDH3.

As used herein, a gene that is a "homolog" or "homologous" to another gene is generally an ortholog (*i.e.*, a descended from the same ancestral sequence but separated when a species diverges into two separate species) or a paralog (*i.e.*, separated by gene duplication within a genome). Typically, homologous genes encode proteins with a moderate to high sequence identity (*e.g.*, at least about 30%, such as at least about 50%, such as at least about 60%, such as at least about 70%, such as at least about 80%, such as at least about 90%, such as at least about 95%, such as at least about 99%, over at least the catalytically active portion, optionally over the full length) and/or can at least partially substitute for the other protein in terms of function, when transferred from one species into another. Homologs of a particular gene can be identified using publicly available and specialized biological databases, *e.g.*, by the eggNOG, InParanoid, OrthoDB, OrthoMCL, OMA, Roundup, TreeFam, LOFT, Ortholuge, EnsemblCompara GeneTrees and HomoloGene.

A "variant" of a parent or reference enzyme comprises one or more mutations, such as amino acid substitutions, insertions and deletions, as compared to the parent or reference enzyme. Typically, the variant has a high sequence identity to the amino acid sequence of the parent or reference enzyme (*e.g.*, at least about 70%, such as at least about 80%, such as at least about 90%, such as at least about 91%, such as at least about 92%, such as at least about 93%, such as at least abut 94%, such as at least about 95%, such as at least about 96%, such as at least 97%, such as at least about 98%, such as at least about 99%, over at least the catalytically active portion, optionally over the full length, although less than 100% sequence identity).

Unless otherwise stated, the term "sequence identity" for amino acid sequences as used herein refers to the sequence identity calculated as $(n_{ref} - n_{dif})\cdot 100/n_{ref}$, wherein n_{dif} is the total number of non-identical residues in the two sequences when aligned and wherein n_{ref} is the number of residues in one of the sequences. Hence, the amino acid sequence GSTDYTQNWA will have a sequence identity of 80% with the sequence GSTGYTQAWA (n_{dif}=2 and n_{ref}=10). The sequence identity can be determined by conventional methods, *e.g.*, Smith and Waterman, (1981), Adv. Appl. Math. 2:482, by the 'search for similarity' method of Pearson & Lipman, (1988), Proc. Natl. Acad. Sci. USA 85:2444, using the CLUSTAL W algorithm of Thompson *et al.*, (1994), Nucleic Acids Res 22:467380, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group). The BLAST algorithm (Altschul *et al.*, (1990), Mol. Biol. 215:403-10) for which software may be obtained through the National Center for Biotechnology Information www.ncbi.nlm.nih.gov/) may also be used. When using any of the aforementioned algorithms, the default parameters for "Window" length, gap penalty, etc., are used.

A residue in one amino acid sequence which "corresponds to" a specific reference residue in a reference amino acid sequence is the residue which aligns with the reference residue. Residues in ASMTs from various species which align with specific reference residues in human ASMT (hsASMT) can be identified in the alignment in Figure 2.

A "fragment" of a protein comprises at least the part of the protein which is responsible for its function of interest, *e.g.*, in the case of an enzyme, its catalytic part. Typically, a "fragment" comprises a segment corresponding to at least about 30%, such as at least about 50%, such as at least about 60%, such as at least about 70%, such as at least about 80%, such as at least about 90%, such as at least about 95%, of the full length protein.

A "functionally active variant" or "functionally active fragment" comprises mutations and/or deletions, respectively, which do not substantially affect the function of the variant or fragment as compared to the parent or reference protein, and can substitute at least partially for the parent or reference protein in terms of the function of interest. Typically, in the case of an enzyme, a functionally active variant or fragment can be described as a catalytically active variant or fragment, and has a catalytic activity, as determined by a suitable activity assay, which is 80-120%, such as 90%-110%, such as 95%-105%, of that of the parent or reference enzyme (which may in itself be a variant of a native enzyme).

As used herein, "vector" refers to any genetic element capable of serving as a vehicle of genetic transfer, expression, or replication for an exogenous nucleic acid sequence in a host cell. For example, a vector may be an artificial chromosome or a plasmid, and may be

capable of stable integration into a host cell genome, or it may exist as an independent genetic element (*e.g.*, episome, plasmid). A vector may exist as a single nucleic acid sequence or as two or more separate nucleic acid sequences. Vectors may be single copy vectors or multicopy vectors when present in a host cell. Preferred vectors for use in the present invention are expression vector molecules in which one or more functional genes can be inserted into the vector molecule, in proper orientation and proximity to expression control elements resident in the expression vector molecule so as to direct expression of one or more proteins when the vector molecule resides in an appropriate host cell.

Standard recombinant DNA and molecular cloning techniques useful for construction of appropriate expression vectors and other recombinant or genetic modification techniques for practising the invention, are well known in the art and are described by, *e.g.*, Green and Sambrook, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press (Cold Spring Harbor, N.Y.) (2012); by Silhavy, T. J., Bennan, M. L. and Enquist, L. W. Experiments with Gene Fusions; Cold Spring Harbor Laboratory: Cold Spring Harbor, New York, 1984; by Ausubel *et al.*, Short Protocols in Molecular Biology, Current Protocols, John Wiley and Sons (New Jersey) (2002), and references cited herein. Appropriate microbial cells and vectors are available commercially through, for example, the American Type Culture Collection (ATCC), Rockville, Md.

The term "host cell" refers to any cell into which an exogenous nucleic acid sequence can be introduced and expressed, typically via an expression vector. The host cell may, for example, be a wild-type cell isolated from its natural environment, a mutant cell identified by screening, a cell of a commercially available strain, or a genetically engineered cell or mutant cell, comprising one or more other exogenous and/or heterologous nucleic acid sequences than those of the invention.

A "recombinant" cell or host cell as used herein refers to a host cell into which one or more exogenous nucleic acid sequences of the invention have been introduced, typically via transformation of a host cell with a vector.

The term "substrate" or "precursor", as used herein in relation to a specific enzyme, refers to a molecule upon which the enzyme acts to form a product. When used in relation to an exogenous biometabolic pathway, the term "substrate" or "precursor" refers to the molecule(s) upon which the first enzyme of the referenced pathway acts. When referring to an enzyme-catalyzed reaction in a microbial cell, an "endogenous" substrate or precursor is a molecule which is native to or biosynthesized by the microbial cell, whereas an "exogenous" substrate or precursor is a molecule which is added to the microbial cell, via a medium or the like.

Enzymes referred to herein can be classified on the basis of the handbook Enzyme Nomenclature from NC-IUBMB, 1992), see also the ENZYME site at the internet: http://www.expasy.ch/enzyme/. This is a repository of information relative to the nomenclature of enzymes, and is primarily based on the recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUB-MB). It describes each type of characterized enzyme for which an EC (Enzyme Commission) number has been provided (Bairoch A., The ENZYME database, 2000, Nucleic Acids Res 28:304-305). The IUBMB Enzyme nomenclature is based on the substrate specificity and occasionally on their molecular mechanism.

Specific embodiments of the invention

Preferred ASMT variants are described below, together with other enzymes in preferred biosynthetic pathways for production of melatonin in recombinant microbial cells (see, *e.g.*, **Figure 1**).

Acetylserotonin O-methyltransferase (ASMT)

ASMT is typically classified as EC 2.1.1.4. Sources of nucleic acid sequences encoding a native ASMT into which the mutations described herein can be introduced include any species where the encoded gene product is capable of catalyzing the referenced reaction, including humans and other mammalian and non-mammalian animals, plants, etc. ASMTs particularly suitable for *E. coli* and other microbial host cells include those whose amino acid sequence are shown in **Figure 3**, *i.e.*, *Homo sapiens, Ocimum basilicum, Bos Taurus, Takifugu rubripes, Macaca mulatta* and *Elephantulus edwardii* ASMT. Particularly preferred is hsASMT.

Examples of nucleic acids encoding ASMT for use in aspects and embodiments of the present invention include, but are not limited to, those encoding the ASMTs listed in **Table 1**, as well as functionally active variants, homologs and fragments thereof. In one embodiment, the ASMT is hsASMT or a functionally (catalytically) active fragment or variant thereof.

Functional fragments and variants of ASMT enzymes are known in the art. For example, to increase heterologous expression in *E. coli* and/or the enzyme stability, the ASMT sequence can be truncated to remove portions not needed for its catalytic activity while preserving the catalytic core of the enzyme, *e.g.*, by 1, 2, 3, 4, 5, 10, 20 or more amino acid residues from the C, terminal, N-terminal, or both. Other ASMT sequences can be similarly truncated to create functionally active fragments or variants comprising the catalytic core.

Many naturally occurring variants of human ASMT are known, though primarily variants in which the mutation has either no significant effect on catalytic activity or reduces or abolishes the catalytic activity. For example, in the UniProt entry for hsASMT (P46597; accessed on April 13, 2016), N13H, K81E and R111K were described as having "no effect on enzyme activity;" E61Q, P243L, I269M, C273S, G278A and V305M are described as "reduced enzyme activity;" and N17K, V171M, D210G, Y248H, R291Q and L298F are described as "nearly abolishes enzyme activity." Accordingly, ASMT variants of the present invention do not comprise N17K, V171M, D210G, Y248H, R291Q and L298F mutations, and do not comprise E61Q, P243L, I269M, C273S, G278A and V305M as the sole mutation. In one embodiment, the ASMT variant does not comprise any of N17K, V171M, D210G, Y248H, R291Q, L298F, E61Q, P243L, I269M, C273S, G278A and V305M

As described herein, the ASMT variants provided by the invention comprise a mutation in at least one residue located in a segment corresponding to residues A258-T272 of hsASMT, the mutation providing for an increased catalytic activity. In one embodiment, the ASMT variant comprises only one mutation in the segment in question. Additionally or alternatively, the ASMT variant may comprise one or more mutations in the segments corresponding to P241-Y248, D259-H271 and T307-Q310, the mutation providing for retained or increased ASMT activity. Catalytically active fragments or variants of such ASMT variants are also contemplated, in particular catalytically active fragments or variants in which the increased catalytic activity is not reduced or abolished. In one embodiment, the ASMT comprises only one mutation as compared to the native ASMT amino acid sequence.

In separate and specific embodiments, the ASMT variant comprises a mutation in a residue corresponding to a hsASMT residue listed below, resulting in one of the indicated amino acid substitutions in one, two or all of the indicated amino acid residues:

A258 => E
G260 => D, N, L, or I
T272 => A or G

Particularly contemplated are variants of the ASMTs listed in **Table 1** and/or in Figure 5 of Botros *et al.* (2013), in particular SEQ ID NOS:1-6 or other ASMTs where at least one residue adjacent to the residue aligning with hsASMT residues A258, G260 or T272 is the same as the adjacent residue in hsASMT. Preferred mutations include those corresponding to A258E, G260D and T272A in hsASMT. In one embodiment, the ASMT variant, or a catalytically active fragment or variant thereof, comprises an A258E mutation. In one embodiment, the ASMT variant, or a catalytically active fragment or variant thereof, comprises a G260D mutation. In

one embodiment, the ASMT variant, or a catalytically active fragment or variant thereof, comprises a T272A mutation.

Preferred variants are those that have a sequence identity of at least about 80%, such as least about 90%, such as at least about 91%, such as at least about 92%, such as at least about 93%, such as at least abut 94%, such as at least about 95%, such as at least about 96%, such as at least 97%, such as at least about 98%, such as at least about 99%, over at least the catalytically active portion of hsASMT, optionally over the full length of hsASMT (although less than 100% sequence identity to hsASMT).

Nucleic acid sequences and vectors encoding the variant ASMTs, or functionally (catalytically) active fragments or variants thereof, as well as recombinant host cells expressing variant ASMTs from such nucleic acid sequences or vectors, are also contemplated. In a preferred embodiment, the nucleic acid sequence encoding the ASMT is operably linked to a strong promoter such as the Trc promoter, providing for high expression levels of the ASMT in an *E. coli* host.

Suitable assays for testing melatonin production by an ASMT *in vitro* or in a recombinant microbial host cell include the assay used in Example 1 under the heading "*hsASMT variants characterization*" as well as the assay described in Kang *et al.* J. Pineal Res. 2011:50;304-309, which is hereby incorporated by reference in its entirety. For example, to identify a mutation providing for an increased catalytic activity, the following methods can be used:

Introduce the desired genetic change into a gene encoding an ASMT, *e.g.*, from *homo sapiens*, using standard molecular biology techniques (Step 1). Express the ASMT variant in a chosen host cell, *e.g.*, *E. coli* (Step 2). To determine ASMT activity in the *in vivo* host cell system, cultivate the ASMT expressing host cells in the presence of acetylserotonin at a defined concentration, sampling the cell broth periodically during active growth (Step 3a), then determine the levels of accumulated melatonin by known analytical techniques and estimate the turnover rate (Step 4a). To determine ASMT activity *in vitro*, harvest variant ASMT protein from the host cells by means of protein purification (Step 3b), and determine the enzyme kinetics (Step 4b).

Using such assays, or some other assay known in the art for determining ASMT catalytic activity in converting N-acetylserotonin to melatonin, an increased catalytic activity can be identified for ASMT variants providing for a higher turnover rate or a higher total yield in the referenced reaction as compared to a control value, *e.g.*, the turnover rate or total yield of the native ASMT in the same assay. Preferably, using the ASMT variant, the turnover rate or total yield is at least 5% higher, at least 10% higher, at least 25% higher, at least 50%

higher (*i.e.*, at least 1.5-fold), at least 100% higher (*i.e.*, at least 2-fold), at least 150% higher (*i.e.*, at least 2.5-fold) or at least 200% higher (*i.e.*, at least 3-fold) than the control, *e.g.*, the turnover rate or total yield when using the native ASMT.

L-Tryptophan hydroxylase (TPH)

Sources of nucleic acid sequences encoding a TPH include any species where the encoded gene product is capable of catalyzing the referenced reaction, including humans, mammals such as, *e.g.*, mouse, cow, horse, chicken and pig, as well as other animals such as, *e.g.*, the parasite *Schistosoma mansoni*. In humans and, it is believed, in other mammals, there are two distinct TPH alleles, referred to herein as TPH1 and TPH2, respectively. As shown in **Figure 1**, TPHs typically require a cofactor; tetrahydrobiopterin (THB, alternatively abbreviated as BH4). It has been reported, however, that native tetrahydromonapterin (MH4) can replace or substitute for THB as a TPH cofactor (US 2014/0134689 AA).

Examples of nucleic acids encoding L-tryptophan hydroxylase for use in aspects and embodiments of the present invention include, but are not limited to, those encoding the TPHs listed in **Table 1**, as well as functionally active variants, homologs and fragments thereof.

Functional fragments and variants of TPH enzymes are known in the art. For example, to increase heterologous expression in *E. coli* and the enzyme stability, the TPH sequence can be truncated to remove portions not needed for its catalytic activity which preserving the catalytic core of the enzyme. Specific examples of functional fragments of TPH include Met102 to Ser416 of *Oryctolagus cuniculus* TPH (Moran *et al.*, J Biol Chem 1998;273(20): 12259-66) and residues Asp45-Arg471 or Glu147-Thr460 (*i.e.*, E147 to T460) of *Homo sapiens* TPH2, optionally adding an N-terminal methionine residue Other TPH sequences can be similarly truncated to create functionally active fragments or variants comprising the catalytic core. For example, the TPH identified as "*Homo sapiens TPH2, truncated ((45-471)+20)*" in **Table 1** represents a fragment of *Homo sapiens* TPH2 comprising an added heterologous 20-amino acid polypeptide at its C-terminal, and the *Homo sapiens* TPH2 sequence denoted "*Homo sapiens TPH2, insert (+6)*" has a 6-amino acid insert in the N-terminal portion. Any one of these mammalian TPHs, such as *Homo sapiens* TPH2, or a fragment and/or variant thereof, can be used for catalyzing the hydroxylation of tryptophan, *e.g.*, in a recombinant microbial cell. Notably, *Schistosoma mansoni* TPH (see **Table 1**) has advantageous properties with respect to, *e.g.*, solubility, thus enabling no or less truncation of the enzyme sequence. Accordingly, *Schistosoma mansoni* TPH, or a functionally active fragment and/or variant thereof, can also be used.

Assays for measuring L-tryptophan hydroxylase activity *in vitro* are well-known in the art (see, *e.g.*, Winge *et al.*, Biochem J, 2008;410:195-204 and Moran *et al.*, 1998).

In the recombinant host cell, the L-tryptophan hydroxylase is typically sufficiently expressed so that an increased level of 5HTP production from L-tryptophan can be detected as compared to the microbial host cell prior to transformation with the TPH, optionally in the presence of added THB cofactor and/or tryptophan substrate. Alternatively, the expression level of the specific TPH enzyme can be evaluated by proteomic analysis, according to methods known in the art. In a preferred embodiment, the nucleic acid sequence encoding the TPH is operably linked to a strong promoter such as the Trc promoter, providing for high expression levels of the TPH.

5HTP decarboxylase

The last step in the serotonin biosynthesis via a 5HTP intermediate, the conversion of 5HTP to serotonin, is in animal cells catalyzed by a 5HTP decarboxylase, which is an aromatic L-amino acid decarboxylase (AADC) typically classified as EC 4.1.1.28. Suitable 5HTP decarboxylases include any tryptophan decarboxylase (TDC) capable of catalyzing the referenced reaction. TDC participates in the plant serotonin biosynthesis pathway, where tryptophan decarboxylase (TDC) catalyzes the conversion of tryptophan to tryptamine, which is then converted into serotonin in a reaction catalyzed by tryptamine 5-hydroxylase (T5H). TDC likewise belongs to the aromatic amino acid decarboxylases categorized in EC 4.1.1.28, and can be able to convert 5HTP to serotonin and carbon dioxide (see, *e.g.*, Park *et al.*, Biosci. Biotechnol. Biocem. 2008;72(9):2456-2458.2008, and Gibson *et al.*, J. Exp. Bot. 1972;23(3):775-786), and thus function as a 5HTP decarboxylase. Exemplary nucleic acids encoding ADDC enzymes for use in aspects and embodiments of the present invention include, but are not limited to, those encoding the 5HTP decarboxylases listed in **Table 1**, as well as functionally active variants, homologs and fragments thereof. In some embodiments, particularly where it is desired to also promote serotonin formation from a tryptamine substrate in the same recombinant cell, an enzyme capable of catalyzing both the conversion of tryptophan to tryptamine and the conversion of 5HTP to serotonin can be used. For example, rice TDC and tomato TDC can function also as a 5HTP decarboxylase, an activity which can be promoted by the presence of pyridoxal phosphate (*e.g.*, at a concentration of about 0.1 mM) (Park *et al.*, 2008). Preferred, non-limiting sources of 5HTP decarboxylase include *Candidatus Koribacter versatilis* Ellin345, *Draconibacterium orientale and Verrucosispora maris* (**Table 1**).

Suitable assays for testing serotonin production by a 5HTP decarboxylase in a recombinant microbial host cell are provided in, or can be adapted from, *e.g.*, Park *et al.* (2008) and Park

et al., Appl Microbiol Biotechnol 2011;89(5):1387-1394. For example, these assays can be adapted to test serotonin production by a 5HTP decarboxylase (*e.g.*, a TDC), either from 5HTP or, in case the microbial cell comprises an L-tryptophan hydroxylase, from L-tryptophan (or simply a carbon source). In one exemplary embodiment, the recombinant microbial cell produces at least 5%, such as at least 10%, such as at least 20%, such as at least 50%, such as at least 100% or more serotonin than the recombinant cell without transformation with 5HTP decarboxylase enzyme, *i.e.*, a background value.

Serotonin acetyltransferase (AANAT)

In one aspect, the recombinant microbial cell further comprises an exogenous nucleic acid sequence encoding a serotonin acetyltransferase, also known as serotonin –N-acetyltransferase, arylalkylamine N-acetyltransferase and AANAT, and typically classified as EC 2.3.1.87. AANAT catalyzes the conversion of acetyl-CoA and serotonin to CoA and N-acetylserotonin (**Figure 1**). Exemplary nucleic acids encoding AANAT enzymes for use in aspects and embodiments of the present invention include, but are not limited to, those encoding the AANATs shown in **Table 1**, as well as functionally active variants, homologs or fragments thereof. Suitable assays for testing N-acetylserotonin production by an AANAT in a recombinant microbial host cell are described in, *e.g.*, Thomas *et al.*, Analytical Biochemistry 1990;184:228-34.

Table 1 – Examples of enzymes and amino acid sequences

Name (EC #)	**Species**	**NCBI or UniProtKB[1] accession No. (SEQ ID)**
acetylserotonin O-methyltransferase (EC 2.1.1.4) (ASMT)	*Homo sapiens*	P46597-1, v1 **(1)**
	Ocimum basilicum	Q9XGV9-1, v1 **(2)**
	Bos taurus	P10950-1, v2 **(3)**
	Takifugu rubripes	XP_011609423.1 **(4)**
	Macaca mulatta	NP_001028112.1 **(5)**
	Elephantulus edwardii	XP_006902482.1 **(6)**
	Oryza sativa	XP_015610997.1 **(7)**
	Rattus norvegicus	NP_653360.2 **(8)**
	Gallus gallus	NP_990674.1 **(9)**
	Chromobacterium violaceum	WP_011135808.1 **(10)**
	Desulfotomaculum kuznetsovii DSM 6115	YP_004515712.1 **(11)**
	Xenopus (Silurana) tropicalis	NP_001011409.1 **(12)**
	Pseudomonas fluorescens	WP_019095725.1 **(13)**
	Candidatus Solibacter usitatus	WP_011682595.1 **(14)**
	Fenneropenaeus chinensis	AAZ66373.1 **(15)**
	Arabidopsis thaliana	NP_200227.1 **(16)**
L-tryptophan hydroxylase (EC 1.14.16.4) (TPH)	*Oryctolagus cuniculus TPH1*	P17290-1, v2
	Homo sapiens TPH1	NP_004170.1
	Homo sapiens TPH2	NP_775489.2
	Gallus gallus	NP_990287.1
	Mus musculus	NP_033440.1
	Equus caballus	NP_001075252.1

	Schistosoma mansoni *Homo sapiens TPH2, insert (+6)* *Homo sapiens TPH2, truncated ((45-471)+20)* *Homo sapiens TPH2, truncated (45-471)* *Homo sapiens TPH2, truncated (146-460)*	AAD01923.1 **(17)** **(18)** **(19)** **(20)**
5HTP decarboxylase (EC 4.1.1.28) (ADDC)	*Acidobacterium capsulatum* *Rattus norwegicus* *Sus scrofa* *Homo sapiens* *Capsicum annuum* *Drosophila caribiana* *Maricaulis maris* (strain MCS10) *Oryza sativa* subsp. Japonica *Pseudomonas putida* S16 *Catharanthus roseus*	WP_015898075.1 XP_006251536.1 NP_999019.1 P20711-1, v2 NP_001312016.1 AAM80956.1 ABI65701.1 XP_015648768.1 WP_013972057.1 P17770-1, v1
serotonin acetyltransferase (EC 2.3.1.87 or 2.3.1.5) (AANAT)	*Chlamydomonas reinhardtii* *Bos Taurus, optionally with A55P mutation* *Gallus gallus* *Homo sapiens* *Mus musculus* *Oryctolagus cuniculus* *Ovis aries*	BAH10512.1 DAA18183.1 NP_990489.1 NP_001079.1 XP_011246971.1 XP_008249128.1 NP_001009461.1

Variants or homologs of any one or more of the enzymes and other proteins listed in **Table 1**, having the referenced activity and a sequence identity of at least 30%, such as at least 50%, such as at least 60%, such as at least 70%, such as at least 80%, such as at least 90%, such as at least 95%, such as at least 99%, over at least the catalytically active portion, optionally over the full length, of the reference amino acid sequence, are also contemplated. The variant or homolog may comprise, for example, 2, 3, 4, 5 or more, such as 10 or more, amino acid substitutions, insertions or deletions as compared to the reference amino acid sequence. In particular conservative substitutions are considered. These are typically within the group of basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagine), hydrophobic amino acids (leucine, isoleucine and valine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, threonine and methionine). Amino acid substitutions which do not generally alter specific activity are known in the art and are described, for example, by H. Neurath and R. L. Hill, 1979, In: The Proteins, Academic Press, New York. The most commonly occurring exchanges are Ala to Ser, Val to Ile, Asp to Glu, Thr to Ser, Ala to Gly, Ala to Thr, Ser to Asn, Ala to Val, Ser to Gly, Tyr to Phe, Ala to Pro, Lys to Arg, Asp to Asn, Leu to Ile, Leu to Val, Ala to Glu, and Asp to Gly. Homologs, such as orthologs or paralogs, having the desired activity can be identified in the same or a related animal or microbial species using the reference sequences provided and appropriate activity testing. Specific enzyme variants are exemplified herein.

A nucleic acid sequence encoding an enzyme or other protein activity listed in **Table 1**

may encode an amino acid sequence that is homologous (*i.e.*, native) or heterologous to the recombinant host cell in question.

Vectors

Also provided are vectors comprising nucleic acid sequences according to the above aspects and embodiments, *e.g.*, encoding an ASMT variant and, optionally, one or more of a TPH, a 5HTP decarboxylase and an AANAT and/or enzymes for biosynthesizing or regenerating co-factors needed for ASMT and TPH.

The specific design of the vector depends on, *e.g.*, whether host cell already endogenously produces sufficient amounts of one or more of the enzymes or cofactors. For example, in an *E. coli* host cell, it may not be necessary to introduce the nucleic acid sequence encoding a GCH1 (FolE) sequence exogenously, in case sufficient amounts of the enzyme is expressed from the native gene or in case the endogenous gene is upregulated. Additionally, for transformation of a particular host cell, two or more vectors with different combinations of the enzymes used in the present invention can be applied. Accordingly, the nucleic acid sequences encoding the ASMT variant and the one or more additional enzymes may be located on the same vector, or on two or more different vectors. The vector can be a plasmid, phage vector, viral vector, episome, an artificial chromosome or other polynucleotide construct, and may, for example, include one or more selectable marker genes and appropriate expression control sequences.

Generally, regulatory control sequences are operably linked to the encoding nucleic acid sequences, and include constitutive, regulatory and inducible promoters, transcription enhancers, transcription terminators, and the like which are well known in the art. The encoding nucleic acid sequences can be operationally linked to one common expression control sequence or linked to different expression control sequences, such as one inducible promoter and one constitutive promoter.

The procedures used to ligate the various regulatory control and marker elements with the encoding nucleic acid sequences to construct the vectors of the present invention are well known to one skilled in the art (see, *e.g.*, Sambrook *et al.*, 2012, supra). In addition, methods have recently been developed for assembling of multiple overlapping DNA molecules (Gibson *et al.*, 2008) (Gibson *et al.*, 2009) (Li & Elledge, 2007), allowing, *e.g.*, for the assembly multiple overlapping DNA fragments by the concerted action of an exonuclease, a DNA polymerase and a DNA ligase.

The promoter sequence is typically one that is recognized by the intended host cell. For an *E.coli* host cell, suitable promoters include, but are not limited to, the lac promoter, the T7 promoter, pBAD, the tet promoter, the Lac promoter, the Trc promoter, the Trp promoter,

the recA promoter, the λ (lamda) promoter, and the PL promoter. Preferred promoters include the Trc promoter. For *Streptomyces* host cells, suitable promoters include that of *Streptomyces coelicolor agarase* (dagA). For a Bacillus host cell, suitable promoters include the sacB, amyL, amyM, amyQ, penP, xylA and xylB. Other promoters for bacterial cells include prokaryotic beta-lactamase (Villa-Kamaroff *et al.*, 1978, Proceedings of the National Academy of Sciences USA 75: 3727-3731), and the tac promoter (DeBoer *et al.*, 1983, Proceedings of the National Academy of Sciences USA 80: 21-25). For an S. cerevisiae host cell, useful promoters include the TEF1, HXT7, TDH3, ENO-1, GAL1, ADH1, ADH2, GAP, TPI, CUP1, PHO5 and PGK, such as PGK1 promoters. Other useful promoters for yeast host cells are described by Romanos *et al.*, 1992, Yeast 8: 423-488. Still other useful promoters for various host cells are described in "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242: 74-94; and in Sambrook *et al.*, 2012, *supra*.

In one embodiment, one or more or all of the exogenous nucleic acids is each under the control of a strong promoter, *e.g.*, each separately selected from Trc, lac, lacUV5, Trp, T7, trac and PL promoter in an *E. coli* host cell, and each separately selected from PGK1, TEF1, HXT7 and TDH3 in an *S. cerevisiae* host cell.

A transcription terminator sequence is a sequence recognized by a host cell to terminate transcription, and is typically operably linked to the 3' terminus of an encoding nucleic acid sequence. Suitable terminator sequences for *E. coli* host cells include the T7 terminator region. Suitable terminator sequences for yeast host cells such as *S. cerevisiae* include CYC1, PGK, GAL, ADH, AOX1 and GAPDH. Other useful terminators for yeast host cells are described by Romanos *et al.*, 1992, *supra*.

A leader sequence is a non-translated region of an mRNA which is important for translation by the host cell. The leader sequence is typically operably linked to the 5' terminus of a coding nucleic acid sequence. Suitable leaders for yeast host cells include S. cerevisiae ENO-1, PGK, alpha-factor, ADH2/GAP, TEF, and Kozak sequence.

A polyadenylation sequence is a sequence operably linked to the 3' terminus of a coding nucleic acid sequence which, when transcribed, is recognized by the host cell as a signal to add polyadenosine residues to transcribed mRNA. Useful polyadenylation sequences for yeast host cells are described by Guo and Sherman, 1995, Mol Cell Biol 15: 5983-5990.

A signal peptide sequence encodes an amino acid sequence linked to the amino terminus of an encoded amino acid sequence, and directs the encoded amino acid sequence into the cell's secretory pathway. In some cases, the 5' end of the coding nucleic acid sequence may inherently contain a signal peptide coding region naturally linked in translation reading frame, while a foreign signal peptide coding region may be required in other cases. Useful signal peptides for yeast host cells can be obtained from the genes for *S. cerevisiae* alpha-factor and invertase. Other useful signal peptide coding regions are described by Romanos *et al.*, 1992, supra. An exemplary signal peptide for an *E. coli* host cell can be obtained from alkaline phosphatase. For a Bacillus host cell, suitable signal peptide sequences can be

obtained from alpha-amylase and subtilisin. Further signal peptides are described by Simonen and Palva, 1993, Microbiological Reviews 57: 109-137.

It may also be desirable to add regulatory sequences which allow the regulation of the expression of the polypeptide relative to the growth of the host cell. Examples of regulatory systems are those which cause the expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Regulatory systems in prokaryotic systems include the lac, tec, and tip operator systems. For example, one or more promoter sequences can be under the control of an IPTG inducer, initiating expression of the gene once IPTG is added. In yeast, the ADH2 system or GAL1 system may be used. Other examples of regulatory sequences are those which allow for gene amplification. In eukaryotic systems, these include the dihydrofolate reductase gene which is amplified in the presence of methotrexate, and the metallothionein genes which are amplified with heavy metals. In these cases, the respective encoding nucleic acid sequence would be operably linked with the regulatory sequence.

The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. The vectors may be linear or closed circular plasmids. The vector may also be an autonomously replicating vector, *i.e.*, a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, *e.g.*, a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one which, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, a single vector or plasmid or two or more vectors or plasmids which together contain the total DNA to be introduced into the genome of the host cell, or a transposon may be used.

The vectors of the present invention preferably contain one or more selectable markers which permit easy selection of transformed cells. The selectable marker genes can, for example, provide resistance to antibiotics or toxins, complement auxotrophic deficiencies, or supply critical nutrients not in the culture media, and/or provide for control of chromosomal integration. Examples of bacterial selectable markers are the dal genes from Bacillus subtilis or Bacillus licheniformis, or markers which confer antibiotic resistance such as ampicillin, kanamycin, chloramphenicol, or tetracycline resistance. Suitable markers for yeast host cells are ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3.

The vectors of the present invention may also contain one or more elements that permit integration of the vector into the host cell genome or autonomous replication of the vector in the cell independent of the genome. For integration into the host cell genome, the vector may rely on an encoding nucleic acid sequence or other element of the vector for integration into the genome by homologous or nonhomologous recombination. Alternatively, the vector may contain additional nucleotide sequences for directing integration by homologous

recombination into the genome of the host cell at a precise location(s) in the chromosome(s). To increase the likelihood of integration at a precise location, the integrational elements should preferably contain a sufficient number of nucleic acids, such as 100 to 10,000 base pairs, preferably 400 to 10,000 base pairs, and most preferably 800 to 10,000 base pairs, which have a high degree of identity with the corresponding target sequence to enhance the probability of homologous recombination. The integrational elements may be any sequence that is homologous with the target sequence in the genome of the host cell. The integrational elements may, for example, non-encoding or encoding nucleotide sequences. The vector may be integrated into the genome of the host cell by non-homologous recombination.

For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question. The origin of replication may be any plasmid replicator mediating autonomous replication which functions in a cell. The term "origin of replication" or "plasmid replicator" is defined herein as a nucleotide sequence that enables a plasmid or vector to replicate *in vivo*. Examples of bacterial origins of replication are the origins of replication of plasmids pBR322, pUC19, pACYC177, and pACYC184 permitting replication in *E. coli*, and pUB1 10, pE194, pTA1060, and pAMβi permitting replication in Bacillus. Examples of origins of replication for use in a yeast host cell are the 2 micron origin of replication, ARS1, ARS4, the combination of ARS1 and CEN3, and the combination of ARS4 and CEN6.

More than one copy of the nucleic acid sequence encoding the enzyme or protein of interest may be inserted into the host cell to increase production of the gene product. An increase in the copy number of the encoding nucleic acid sequence can be obtained by integrating at least one additional copy of the sequence into the host cell genome or by including an amplifiable selectable marker gene with the nucleic acid sequence where cells containing amplified copies of the selectable marker gene, and thereby additional copies of the sequence, can be selected for by cultivating the cells in the presence of the appropriate selectable agent.

Recombinant host cells

Recombinant host cells expressing ASMT variants, optionally from the above-described nucleic acid sequences or vectors, are also provided. Suitable host cells for expressing ASMT variants include, for example, mammalian and microbial cells. Once expressed, the ASMT variants can, optionally, be retrieved and purified from the host cells or the cell medium in which the host cells are grown.

The recombinant host cell can also be capable of producing melatonin. For example, mammalian cells comprising a native biosynthetic pathway for melatonin can be transformed with a vector expressing an ASMT variant according to the present invention, under the

control of a promoter suitable for the selected mammalian host cell. Alternatively, the recombinant host cell of the invention can be prepared from any host cell by introducing heterologous or overexpressing endogenous enzymes of the necessary biometabolic pathways, using recombinant techniques well known in the art and cited elsewhere herein.

The host cell is preferably tryptophan autotrophic (*i.e.*, capable of endogenous biosynthesis of L-tryptophan), grows on synthetic medium with suitable carbon sources, and expresses a suitable RNA polymerase (such as, *e.g.*, T7 polymerase). In all known microorganisms, tryptophan production takes place via a single metabolic pathway (Somerville, R. L., Herrmann, R. M., 1983, Amino acids, Biosynthesis and Genetic Regulation, Addison-Wesley Publishing Company, U.S.A.: 301-322 and 351-378; Aida *et al.*, 1986, Bio-technology of amino acid production, progress in industrial microbiology, Vol. 24, Elsevier Science Publishers, Amsterdam: 188-206). Tryptophan can also or alternatively be added to the medium of a recombinant host cell comprising TPH, ASMT and other enzymes providing for a melatonin biosynthetic pathway.

The recombinant host cell is typically capable of biosynthesizing and/or regenerating the cofactors used by the enzymes in the melatonin biosynthesis pathway. In particular, the recombinant host cell is preferably capable of biosynthesizing, regenerating, or bio-synthesizing and regenerating, one or more cofactors for TPH, AANAT and ASMT (**Figure 1**).

To provide cofactor for TPH-catalyzed hydroxylation of tryptophan, the recombinant host cell is preferably capable of biosynthesizing one or both of THB and MH4 via endogenous or heterologous (introduced) pathways. For example, endogenous pathways for THB biosynthesis are present in mammalian cells. Microbial cells generally do not biosynthesize THB endogenously, but it has been reported that the endogenous compound MH4 may substitute for or replace THB as cofactor for TPH in such cells (US 2014/134689 AA; University of California). GTP cyclohydrolase I (such as, *e.g.* FolE) -catalyzed pterin biosynthesis resulting in MH4 takes place in many organisms including both prokaryotes and eukaryotes (see, *e.g.* Figure 9 of US 2014/134689 AA). So, for example, in one embodiment, the microbial host cell is an *E. coli* cell comprising the endogenous enzymes folE, folX, P-ase, and folM, optionally upregulated or expressed from one or more endogenous vectors.

Alternatively, enzymes of biosynthetic pathways for producing and/or regenerating THB can be introduced recombinantly, as described in WO 2013/127914 A1, WO 2013/127915 A1 and WO 2015/032911 A1 (Danmarks Tekniske Universitet) and in US 2014/134689 AA (University of California), all of which hereby incorporated by reference in their entireties. Briefly, in one embodiment, the recombinant cell comprises an exogenous pathway producing THB from GTP and herein referred to as "first THB pathway", comprising a GTP cyclohydrolase I (GCH1), a 6-pyruvoyl-tetrahydropterin synthase (PTPS), and a sepiapterin

reductase (SPR) (see **Figure 1**). The addition of such a pathway to microbial cells such as *E. coli* (JM101 strain), *S. cerevisiae* (KA31 strain) and *Bacillus subtilis* (1A1 strain (TrpC2)) has also been described in, *e.g.*, U.S. 7,807,421. In one embodiment, the recombinant cell comprises a pathway producing THB by regenerating THB from HTHB, herein referred to as "second THB pathway", comprising a 4a-hydroxytetrahydrobiopterin dehydratase (PCBD1) and a 6-pyruvoyl-tetrahydropterin synthase (DHPR). As shown in Figure 1, the second THB pathway converts the HTHB formed by the L-tryptophan hydroxylase-catalyzed hydroxylation of L-tryptophan back to THB, thus allowing for a more cost-efficient 5HTP synthesis. In one embodiment, the recombinant host cell comprises enzymes of both the first and second THB pathways. Non-limiting and exemplary nucleic acids encoding enzymes of the first and second THB pathways for use in aspects and embodiments of the present invention include those shown in Table 1 of WO 2015/032911 A1, which is hereby specifically incorporated by reference, including the actual amino acid sequences referred to in the table as SEQ ID numbers.

Most types of host cells (*e.g.*, mammalian host cells, yeast host cells such as *S. cerevisiae*, bacteria such as *E. coli*, etc.) are capable of producing and regenerating acetyl-CoA and SAM; the cofactors for AANAT and ASMT, respectively.

AcCoA serves as a metabolic cofactor in the AANAT reaction, but is also part of other, endogenous pathways in, *e.g.*, microbial cells.

SAM is a principal methyl donor in various intracellular transmethylation reactions. It is synthesized in the cell through SAM synthetase from methionine and ATP, and natively generated through the SAM cycle, which consists of a methyl transferase, an S-adenosyl-L-homocysteine hydrolase, a folate transferase, and an S-adenosyl-methionine synthetase (Lee *et al.*, Korean J. Chem. Eng. 2010, 27, 587-589). Accordingly, in the ASMT-catalyzed, last reaction in the production of melatonin from L-tryptophan, N-acetylserotonin and SAM are converted to melatonin and SAH. SAH can then be recycled back to SAM via the SAM-cycle in microbial cells where the S-adenosyl-L-methionine cycle is native (or exogenously added) and constitutively expressed, such as, *e.g.*, in *E.coli*. The enzymes of such native pathways can also, in needed, be upregulated or expressed from an exogenously introduced vector, using well-known recombinant techniques known in text books referenced elsewhere herein. Non-limiting and exemplary nucleic acids encoding enzymes of the SAM cycle for use in aspects and embodiments of the present invention include those shown in Table 1 of WO 2015/032911 A1, which is hereby specifically incorporated by reference, including the actual amino acid sequences referred to in the table as SEQ ID numbers.

The recombinant host cell is typically prepared by introducing into the host cell, typically via transformation, one or more vectors according to any preceding embodiment, using standard methods known in the art and cited elsewhere herein. The introduction of a vector into a

bacterial host cell may, for instance, be effected by protoplast transformation (see, *e.g.*, Chang and Cohen, 1979, Molecular General Genetics 168: 111-115), using competent cells (see, *e.g.*, Young and Spizizen, 1961 , Journal of Bacteriology 81 : 823-829, or Dubnau and Davidoff-Abelson, 1971 , Journal of Molecular Biology 56: 209-221), electroporation (see, *e.g.*, Shigekawa and Dower, 1988, Biotechniques 6: 742-751), or conjugation (see, *e.g.*, Koehler and Thome, 1987, Journal of Bacteriology 169: 5771-5278).

As described above, the vector, once introduced, may be maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector.

Preferably, for transformation of an *E. coli* or other bacterial host cell, the vectors are designed as follows: A *lac* promoter is used to control the expressions of a gene or an artificial operon containing up to three genes connected with a linker sequence, in order to express the genes at a suitable level so that the introduction of heterologous genes/pathways do not overdraw substrates or energy in the host cell. In one particular embodiment, the recombinant microbial cell, preferably derived from a bacterial cell, is transformed according to a strategy outlined in the Examples.

Preferably, for transformation of a yeast host cell such as *S. cerevisiae*, the heterologous genes are integrated onto chromosome using a homologous recombination based method (Mikkelsen *et al.*, 2012). As compared with gene expression based on plasmids, the chromosomal integrated genes can be expressed with higher fidelity and resulted in better protein translation, in particular for multiple gene co-expression systems.

The transformation can be confirmed using methods well known in the art. Such methods include, for example, nucleic acid analysis such as Northern blots or polymerase chain reaction (PCR) amplification of mRNA, or immunoblotting for expression of gene products, or other suitable analytical methods to test the expression of an introduced nucleic acid sequence or its corresponding gene product, including those referred to above and relating to measurement of 5HTP production. Expression levels can further be optimized to obtain sufficient expression using methods well known in the art and as disclosed herein.

In one embodiment, the recombinant host cell has been modified so as to downregulate or delete a native gene encoding a cyclopropane fatty acyl phospholipid synthase. As shown in Example 1, *E. coli* cells carrying melatonin production pathway genes stopped melatonin production when mutations occurred in the *cfa* gene, using a methyltransferase selection system. Without being limited to theory, downregulating or deleting a gene corresponding to the *cfa* gene in a microbial host cell into which a biosynthetic pathway for melatonin-production according to Figure 1 has been introduced, improves SAM availability to ASMT, thus improving the yield of melatonin. The amino acid sequence of the Cfa protein and the location of the *cfa* gene in the *E. coli* genome is known in the art (see NCBI Reference Sequence: NP_416178.1 and references cited therein). Orthologs to the *cfa* gene in *E. coli* exist in, *e.g.*, *C. glutamicum* ATCC 13032 (*cma*).

In a preferred embodiment, the host cell is a microbial cell. The microbial host cell for use in the present invention is typically unicellular and can be, for example, a bacterial cell, a yeast host cell, a filamentous fungal cell, or an algeal cell. Examples of suitable host cell genera include, but are not limited to, *Acinetobacter, Agrobacterium, Alcaligenes, Anabaena, Aspergillus, Bacillus, Bifidobacterium, Brevibacterium, Candida, Chlorobium, Chromatium, Corynebacteria, Cytophaga, Deinococcus, Enterococcus, Erwinia, Erythrobacter, Escherichia, Flavobacterium, Hansenula, Klebsiella, Lactobacillus, Methanobacterium, Methylobacter, Methylococcus, Methylocystis, Methylomicrobium, Methylomonas, Methylosinus, Mycobacterium, Myxococcus, Pantoea, Phaffia, Pichia, Pseudomonas, Rhodobacter, Rhodococcus, Saccharomyces, Salmonella, Sphingomonas, Streptococcus, Streptomyces, Synechococcus, Synechocystis, Thiobacillus, Trichoderma, Yarrowia and Zymomonas*.

In one embodiment, the host cell is bacterial cell, *e.g.*, an *Escherichia* cell such as an *Escherichia coli* cell; a *Bacillus* cell such as a *Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus brevis, Bacillus circulans, Bacillus clausii, Bacillus coagulans, Bacillus lautus, Bacillus lentus, Bacillus licheniformis, Bacillus megaterium, Bacillus stearothermophilus, Bacillus subtilis*, or a *Bacillus thuringiensis* cell; or a *Streptomyces* cell such as a *Streptomyces lividans* or *Streptomyces murinus* cell. In a particular embodiment, the recombinant microbial cell is derived from cell of the *Escherichia* genus, such as an *Escherichia coli* cell. In another particular embodiment, the host cell is of an *E. coli* strain selected from the group consisting of K12.DH1 (Proc. Natl. Acad. Sci. USA, volume 60, 160 (1968)), JM101, JM103 (Nucleic Acids Research (1981), 9, 309), JA221 (J. Mol. Biol. (1978), 120, 517), HB101 (J. Mol. Biol. (1969), 41, 459) and C600 (Genetics, (1954), 39, 440).

In one embodiment, the host cell is a fungal cell, such as, *e.g.*, a yeast cell. Exemplary yeast cells include *Candida, Hansenula, Kluyveromyces, Pichia, Saccharomyces, Schizosaccharomyces and Yarrowia* cells. In a particular embodiment, the host cell is an *S. cerevisiae* cell. In another particular embodiment, the host cell is of an *S. cerevisie* strain selected from the group consisting of *S. cerevisiae* KA31, AH22, AH22R-, NA87-11A, DKD-5D and 20B-12, *S. pombe* NCYC1913 and NCYC2036 and *Pichia pastoris* KM71.

In one embodiment, the recombinant microbial is derived from an *Escherichia*, *Saccharomyces*, a *Schizosaccharomyces*, a *Corynebacterium*, a *Bacillus* or a *Streptomyces* cell.

Production of melatonin

The invention also provides a method of producing melatonin, comprising culturing the recombinant microbial cell of any preceding aspect or embodiment in a medium comprising a carbon source. The desired compound can then optionally be isolated or retrieved from the medium, and optionally further purified. Importantly, using a recombinant microbial cell according to the invention, the method can be carried out without adding L-tryptophan, THB,

or both, to the medium.

Also provided is a method of preparing a composition comprising melatonin, comprising culturing the recombinant microbial cell of any preceding aspect or embodiment, isolating and purifying the compound, and adding any excipients to obtain the composition.

Suitable carbon sources include carbohydrates such as monosaccharides, oligosaccharides and polysaccharides. As used herein, "monosaccharide" denotes a single unit of the general chemical formula Cx(H2O)y, without glycosidic connection to other such units, and includes glucose, fructose, xylose, arabinose, galactose and mannose. "Oligosaccharides" are compounds in which monosaccharide units are joined by glycosidic linkages, and include sucrose and lactose. According to the number of units, oligosacchardies are called disaccharides, trisaccharides, tetrasaccharides, pentasaccharides etc. The borderline with polysaccharides cannot be drawn strictly; however the term "oligosaccharide" is commonly used to refer to a defined structure as opposed to a polymer of unspecified length or a homologous mixture. "Polysaccharides" is the name given to a macromolecule consisting of a large number of monosaccharide residues joined to each other by glycosidic linkages, and includes starch, lignocellulose, cellulose, hemicellulose, glycogen, xylan, glucuronoxylan, arabinoxylan, arabinogalactan, glucomannan, xyloglucan, and galactomannan. Other suitable carbon sources include acetate, glycerol, pyruvate and gluconate. In one embodiment, the carbon source is selected from the group consisting of glucose, fructose, sucrose, xylose, mannose, galactose, rhamnose, arabinose, fatty acids, glycerine, glycerol, acetate, pyruvate, gluconate, starch, glycogen, amylopectin, amylose, cellulose, cellulose acetate, cellulose nitrate, hemicellulose, xylan, glucuronoxylan, arabinoxylan, glucomannan, xyloglucan, lignin, and lignocellulose. In one embodiment, the carbon source comprises one or more of lignocellulose and glycerol. In one embodiment, the carbon source is a simple carbon source such as glucose, xylose, fructose, arabinose, galactose, mannose, glycerol, acetate, or a mixture of any thereof.

The culture conditions are adapted to the recombinant microbial host cell, and can be optimized to maximize production or melatonin or a related compound by varying culture conditions and media components as is well-known in the art.

For a recombinant *Escherichia coli* cell, exemplary media include LB medium and M9 medium (Miller, Journal of Experiments in Molecular Genetics, 431-433, Cold Spring Harbor Laboratory, New York, 1972), optionally supplemented with one or more amino acids. When an inducible promoter is used, the inductor can also be added to the medium. Examples include the lac promoter, which can be activated by adding isopropyl-beta-thiogalacto-pyranoside (IPTG) and the GAL/BAD promoter, in which case galactose/arabinose can be added. The culturing can be carried out a temperature of about 10 to 40 °C for about 3 to 72 hours, if desired, with aeration or stirring.

For a recombinant *Bacillus* cell, culturing can be carried out in a known medium at about 30 to 40 °C for about 6 to 40 hours, if desired with aeration and stirring. With regard to the

medium, known ones may be used. For example, pre-culture can be carried out in an LB medium and then the main culture using an NU medium.

For a recombinant yeast cell, Burkholder minimum medium (Bostian, K. L., *et al.* Proc. Natl. Acad. Sci. USA, volume 77, 4505 (1980)), SD medium containing 0.5% of Casamino acid (Bitter, G. A., *et al.*, Proc. Natl. Acad. Sci. USA, volume 81, 5330 (1984), and Delft medium (Verduyn *et al.*, Yeast 1992, 8, 501-517) can be used. The pH is preferably adjusted to about 5-8. For example, a synthetic medium may contain, per litre: (NH4)2SO4, 5 g; KH2PO4, 3 g; MgSO4.7H2O, 0.5 g; EDTA, 15 mg; ZnSO4.7H2O, 4.5 mg; CoCl2.6H2O, 0.3 mg; MnCl2.4H20, 1 mg; CuSO4 5H2O, 0.3 mg; CaCl2.2H2O, 4.5 mg; FeSO4.7H2O, 3 mg; NaMoO4.2H2O, 0.4 mg; H3BO3, 1 mg- KI, 0.1 mg; and 0.025 ml silicone antifoam(BDH). Filter-sterilized vitamins can be added after heat sterilization (120°C), to final concentrations per litre of: biotin, 0.05 mg; calcium pantothenate, 1 mg; nicotinic acid, 1 mg; inositol, 25 mg; thiamine HCl, 1 mg; pyridoxine HCl, 1 mg; and para- aminobenzoic acid, 0.2 mg. The medium can then be adjusted to pH6 with KOH. Culturing is preferably carried out at about 20 to about 40 °C, for about 24 to 84 hours, if desired with aeration or stirring.

In one embodiment, no L-tryptophan is added to the medium. In another embodiment, no L-tryptophan or THB is added to the medium, so that the production of melatonin or its precursors or related compounds rely on substrates biosynthesized in the recombinant host cell.

Using the method for producing melatonin, a melatonin yield of at least about 0.5%, such as at least about 1%, such as at least 5%, such as at least 10%, such as at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80% or at least 90% of the theoretically possible yield can be obtained from a suitable carbon source, such as glucose.

Isolation of melatonin from the cell culture can be achieved, *e.g.*, by separating the compound from the cells using a membrane, using, for example, centrifugation or filtration methods. The product-containing supernatant is then collected. Further purification of the desired compound can then be carried out using known methods, such as, *e.g.*, salting out and solvent precipitation; molecular-weight-based separation methods such as dialysis, ultrafiltration, and gel filtration; charge-based separation methods such as ion-exchange chromatography; and methods based on differences in hydrophobicity, such as reversed-phase HPLC; and the like. In one embodiment, ion-exchange chromatography is used for purification of serotonin. In one embodiment, reverse-phase chromatography is used for separation and/or purification of melatonin. An exemplary method for purification of these indolamines using reversed-phase chromatography is described in Harumi *et al.*, (1996) (J Chromatogr B 675:152-156).

Once a sufficiently pure preparation has been achieved, suitable excipients, stabilizers can optionally be added and the resulting preparation incorporated in a composition for use in preparing a product such as, *e.g.*, a dietary supplement, a pharmaceutical, a cosmeceutical,

or a nutraceutical. For a dietary supplement comprising melatonin, each serving can contain, *e.g.*, from about 0.01 mg to about 100 mg melatonin, such as from about 0.1 mg to about 10 mg, or about 1-5 mg, such as 2-3 mg. Emulsifiers may be added for stability of the final product. Examples of suitable emulsifiers include, but are not limited to, lecithin (*e.g.*, from egg or soy), and/or mono- and di-glycerides. Other emulsifiers are readily apparent to the skilled artisan and selection of suitable emulsifier(s) will depend, in part, upon the formulation and final product. Preservatives may also be added to the nutritional supplement to extend product shelf life. Preferably, preservatives such as potassium sorbate, sodium sorbate, potassium benzoate, sodium benzoate or calcium disodium EDTA are used.

EXAMPLE 1

Media and growth conditions

All strains were maintained at 37℃ in LB (Lennox) Broth (Sigma-Aldrich), 2xYT or M9 minimum media containing 1xM9 minimal salts (BD Difco™), 2 mM $MgSO_4$, 100 µM $CaCl_2$, 500-fold diluted trace minerals (10 g/l $FeCl_3 \cdot 6H_2O$, 2 g/l $ZnSO_4 \cdot 7H_2O$, 0.4 g/l $CuCl_2 \cdot 2H_2O$, 1 g/l $MnSO_4 \cdot H_2O$, 0.6 g/l $CoCl_2 \cdot 6H_2O$, and 1.6 mM EDTA, pH 8.0), 1x ATCC® Vitamin Supplement (ATCC MD-VS™), and 0.2% glucose (w/v).

Plasmids

The wild type human ASMT (hsASMT), codon-optimized for *E. coli* expression, was harbored in the pHM18 plasmid of a P15A origin. The A258E, G260D and T272A variants of hsASMT were constructed by site-directed mutagenesis and were harbored in pHM64, pHM65 and pHM66 derived from pHM18. All hsASMT variants were expressed under a strong Ptrc promoter.

Metabolite analysis by LC-MS

LC-MS data was collected on OrbiTrap Fusion High Resolution Mass Spectrometer system coupled with an Ultimate 3000 UHPLC pump (Thermo, San Jose Ca). Samples were held in the autosampler at a temperature of 10.0°C during the analysis. 1µL Injections of the sample were made onto a Thermo HyperSil Gold PFP HPLC column, with a 3 um particle size, 2.1 mm i.d. and 150 mm long. The column was held at a temperature of 35.0°C. The solvent system used was Solvent A "Water with 0.1% formic acid" and Solvent B "Acetonitrile with 0.1% formic ". The Flow Rate was 1.000 ml/min with an Initial Solvent composition of %A = 95, %B = 5 held until 0.50 min, the solvent composition was then changed following a Linear Gradient until it reached %A = 70.0 and %B = 30.0 at 1.50 min. The solvent composition was then changed following a Linear Gradient until it reached %A = 5.0 and %B = 95.0 at

2.00 min This was held until 2.50 min when the solvent was returned to the initial conditions and the column was re-equilibrated until 3.00 min. The first 0.25 min of the run was diverted to waste using the divert valve, following which the column eluent flowed directly into the Heated ESI probe of the MS which was held at 325°C and a voltage of 3500 V. Data was collected in positive ion mode over the mass range 50 to 1000 m/z at a resolution of 15.000. The other MS settings were as follows, Sheath Gas Flow Rate of 60 units, Cone Gas Flow Rate of 20 units Cone Temp was 275°C.

hsASMT variants characterization

Three point mutations of hsASMT were identified using a methyltransferase engineering platform. The mutations were A258E, G260D and T272A, respectively. All three mutations were re-introduced onto the wild-type hsASMT gene yielding pHM64, pHM65 and pHM66 derived from the wild-type hsASMT carrying pHM18. The resulting plasmids were transformed into *E. coli*. The transformed cells were subjected to an *in vivo* turnover activity assay whereas cells were grown in M9 medium supplemented with 200 mg/l of acetylserotonin at 37°C following by removing cell broth at various time points for exo-metabolites analysis and turnover activity calculations. Turnover activity of the mutants was benchmarked against wild-type hsASMT and results indicated all mutational changes led to an increase in acetylserotonin turnover at 2.6-fold, 2.3-fold and 1.6-fold, respectively (**Figure 2**).

Structural analysis of hsASMT variants

Wild-type human ASMT comprises 345 amino acids. Botros *et al.* (2013) reported two structural states of hsASMT with either s-adenosylmethionine (SAM) bound (PDB: 4a6d) or both SAM and N-acetylserotonin bound (PDB: 4a6e). Structural analysis showed that the hsASMT protein consisted of 16 α-helices and the A258, G260 and T272 residues played a role in positioning the 14^{th} helix. The 14^{th} helix, D259-H271, locates at a strategic position whereas it guards the entry opening of the SAM cofactor via interactions with the adjacent residues (including P241-Y248 and T307-Q310). In particular, without being limited to theory, the three beneficial mutations identified may each have resulted in a widening of the SAM entry opening. For example, a mutation of A258 to Glu (E) could result in a repulsive hydrophilic interaction with E308, hence "pushing" the helix away for enlarged opening. In contrast, the T272A mutation could relax the hydrophobic interaction between L242-E244 so that the helix becomes more flexible. Finally, the mutation of G260 to Asp (D) apparently interacts with D259, the first amino acid of the helix-14. The structural analysis showed that the identified mutations regulated SAM entry and possibly affected affinity of hsASMT towards SAM (*i.e.* a low K_m for SAM). Furthermore, sequence alignment of 6 functional ASMTs showed that the identified residues were less conserved throughout the course of the

evolution than the surrounding residues, suggesting a flexibility to changes in individual organisms to adapt their own cellular SAM environment (**Figure 3**).

Identifying Cfa as a native SAM sink

Using the methyltransferase engineering platform, non-melatonin producers with *cfa* mutations were identified. The specific mutations occurred either within the initial coding region of *cfa* (*e.g.* C5S or C5Y) or its promoter region, indicating changes in expression of the *cfa* gene. Since the *cfa* gene encodes for a SAM-dependent fatty acid synthase, it was concluded that Cfa was the SAM sink for the non-melatonin producers.

LIST OF REFERENCES

Botros *et al.*, J Pineal Res 2013;54:46–57
Kang *et al.*, J. Pineal Res. 2011; 50:304-309
Byeon *et al.*, J. Pineal Res 2016; 60:65-73
UniProtKB - P46597 (ASMT_HUMAN); accessed on 13 April 2016
WO 2013/127914 A1, WO 2013/127915 A1 and WO 2015/032911 A1 (Danmarks Tekniske Universitet)
US 2014/134689 AA (University of California)
WO 2007/052166 (Institut Pasteur)
WO 02/06337 (Shanghai Biowindow Gene Development Inc.)
UniProtKB database entry AOA096MY35
UniProtKB database entry A0A091UVP6
UniProt database entry A0A091GD70
EBI accession no. EMBL:KFQ94517.1

CLAIMS

1. A variant of *Homo sapiens* acetylserotonin O-methyltransferase (hsASMT), the variant having at least 92% sequence identity to hsASMT (SEQ ID NO:1) and comprising one or more mutations in the segment corresponding to residues A258 to T272 in *Homo sapiens* ASMT (SEQ ID NO:1), providing for an increased catalytic activity in converting N-acetylserotonin to melatonin as compared to hsASMT.

2. The variant ASMT of claim 1, having at least 50% increased catalytic activity in converting N-acetylserotonin to melatonin as compared to hsASMT.

3. The variant ASMT of any one of claims 1 and 2, having at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% sequence identity to *Homo sapiens* ASMT (SEQ ID NO:1).

4. The variant ASMT of any one of claims 1 to 3, comprising a mutation in a residue selected from the group consisting of A258, G260, and T272.

5. The variant ASMT of any one of claims 1 to 4, wherein the mutation is an amino acid substitution selected from the group consisting of
   (a) A258E;
   (b) G260D, G260N, G260L, and G260I; and
   (c) T272A and T272G.

6. A variant of hsASMT, the variant comprising SEQ ID NO:1, or a catalytically active fragment thereof, having a A258E, G260D or T272A mutation.

7. A nucleic acid sequence encoding the variant ASMT of any one of the preceding claims.

8. A vector comprising the nucleic acid sequence of claim 7, optionally operably linked to one or more expression control sequences.

9. A recombinant host cell comprising the variant ASMT of any one of claims 1 to 6, the nucleic acid of claim 7 or the vector of claim 8.

10. The recombinant host cell of claim 9, which is a mammalian cell, a bacterial cell, a yeast cell, a filamentous fungal cell or an algal cell.

11. The recombinant host cell of claim 10, which is an *Escherichia cell*.

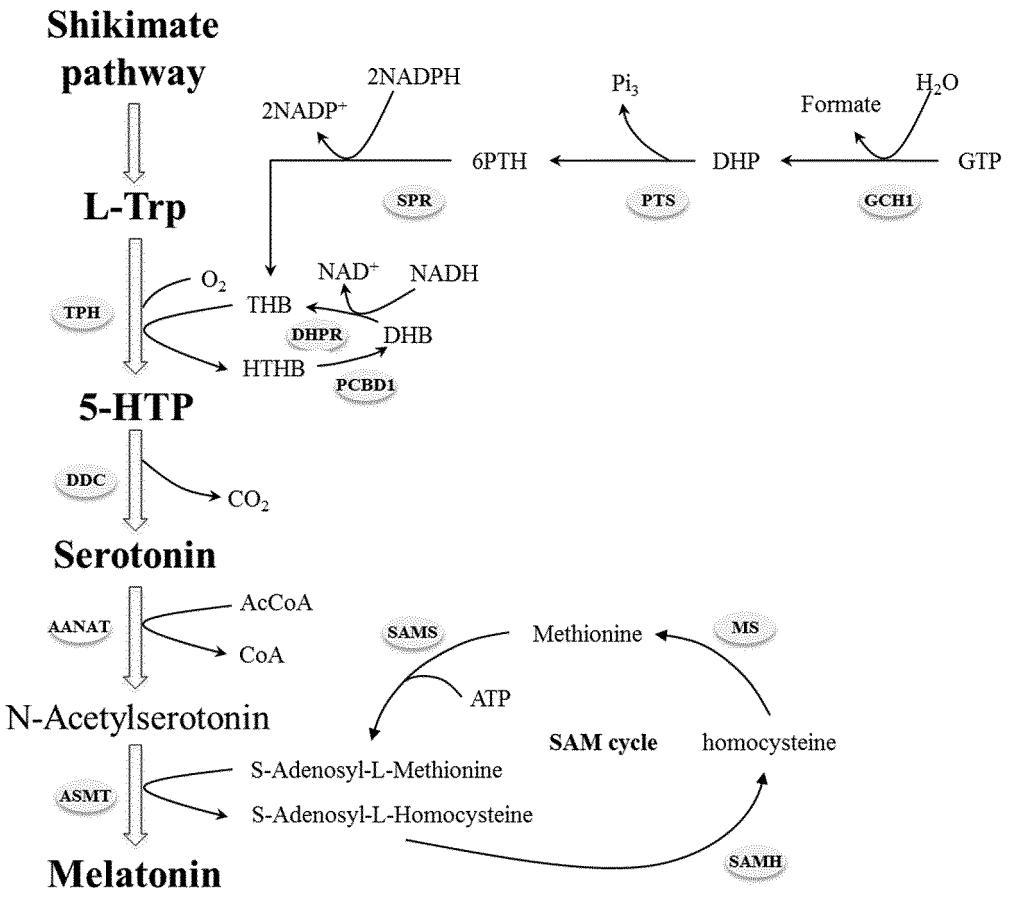
12. The recombinant host cell of any one of claims 9 to 11, comprising a native or heterologous biosynthetic pathway for producing *N*-acetylserotonin and a native or heterologous pathway for recycling S-adenosyl-L-homocysteine (SAH) into S-adenosyl-L-methionine (SAM).

13. The recombinant host cell of any one of claims 9 to 12, comprising a deletion or downregulation of a gene encoding a cyclopropane fatty acyl phospholipid synthase.

14. A recombinant host cell derived from an *E. coli* cell, comprising an ASMT, a native or heterologous biosynthetic pathway for producing *N*-acetylserotonin, a native or heterologous pathway for recycling SAH into SAM, and a deletion or downregulation of the *cfa* gene.

15. A method of producing melatonin, comprising culturing the recombinant host cell of any one of claims 9 to 14 in a medium comprising a carbon source, and, optionally, isolating melatonin.

FIG. 1


Shikimate pathway
L-Trp
TPH
5-HTP
DDC
Serotonin
AANAT
N-Acetylserotonin
ASMT
Melatonin
2NADPH
2NADP+
SPR
6PTH
Pi3
PTS
DHP
Formate
H2O
GCH1
GTP
O2
NAD+
NADH
THB
DHPR
DHB
HTHB
PCBD1
CO2
AcCoA
CoA
SAMS
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S-Adenosyl-L-Homocysteine
SAMH

FIG. 2

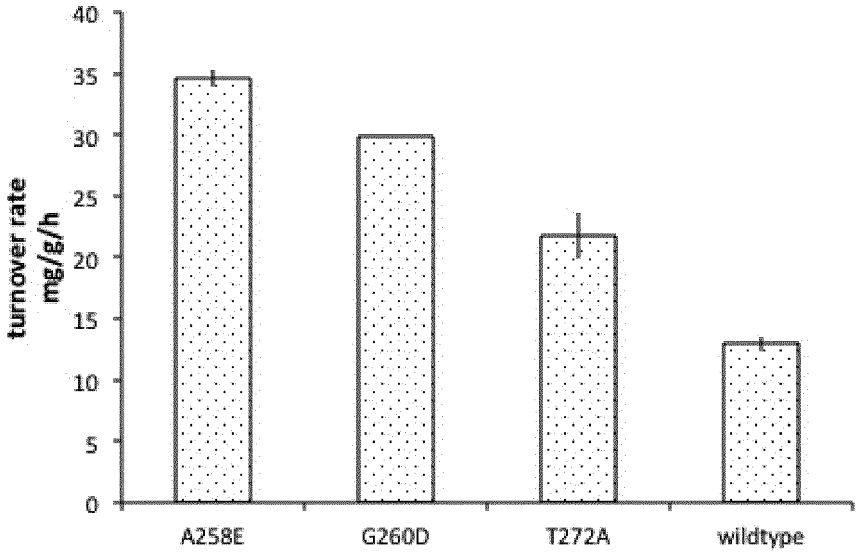
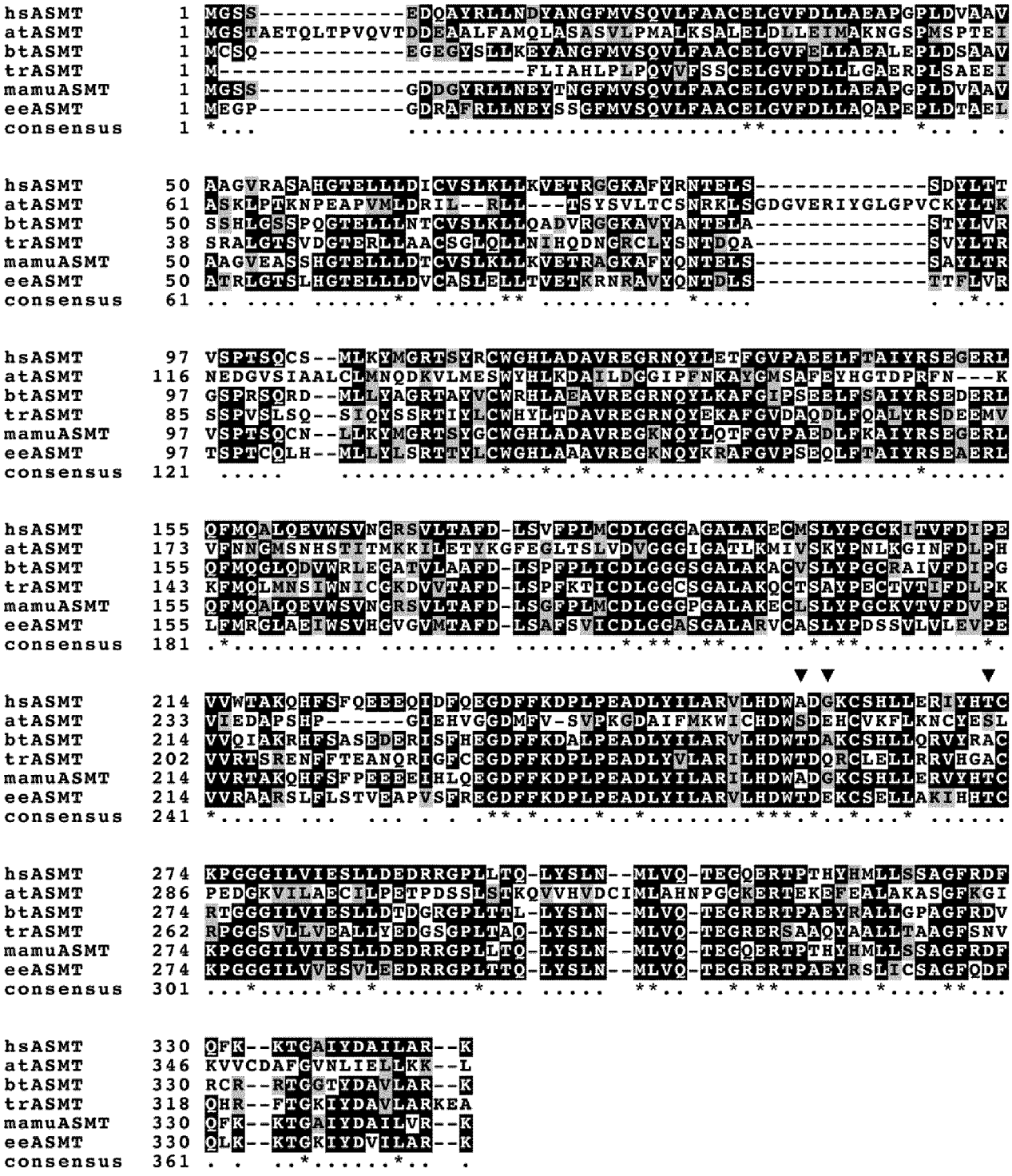

turnover rate mg/g/h
40
35
30
25
20
15
10
5
0
A258E
G260D
T272A
wildtype

FIG. 3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btASMT 1 MCSQ----------EGEGYSLLKEYANGFMVSQVLFAACELGVFELLAEALEPLDSAAV
trASMT 1 M----------FLIAHLPLPQVVFSSCELGVFDLLLGAERPLSAEEI
mamuASMT 1 MGSS----------GDDGYRLLNEYTNGFMVSQVLFAACELGVFDLLAEAPGPLDVAAV
eeASMT 1 MEGP----------GDRAFRLLNEYSSGFMVSQVLFAACELGVFDLLAQAPEPLDTAEL
consensus 1
hsASMT 50 AAGVRASAHGTELLLDICVSLKLLKVETRGGKAFYRNTELS----------SDYLTT
atASMT 61 ASKLPTKNPEAPVMLDRILRLL---TSYSVLTCSNRKLSGDGVERIYGLGPVCKYLTK
btASMT 50 SSHLGSSPQGTELLLNTCVSLKLLQADVRGGKAVYANTELA----------STYLVR
trASMT 38 SRALGTSVDGTERLLAACSGLQLLNIHQDNGRCLYSNTDQA----------SVYLTR
mamuASMT 50 AAGVEASSHGTELLLDTCVSLKLLKVETRAGKAFYQNTELS----------SAYLTR
eeASMT 50 ATRLGTSLHGTELLLDVCASLELLTVETKRNRAVYQNTDLS----------TTFLVR
consensus 61
hsASMT 97 VSPTSQCS--MLKYMGRTSYRCWGHLADAVREGRNQYLETFGVPAEELFTAIYRSEGERL
atASMT 116 NEDGVSIAALCLMNQDKVLMESWYHLKDAILDGGIPFNKAYGMSAFEYHGTDPRFN---K
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trASMT 143 KFMQLMNSIWNICGKDVVTAFD-LSPFKTICDLGGCSGALAKQCTSAYPECTVTIFDLPK
mamuASMT 155 QFMQALQEVWSVNGRSVLTAFD-LSGFPLMCDLGGGPGALAKECLSLYPGCKVTVFDVPE
eeASMT 155 LFMRGLAEIWSVHGVGVMTAFD-LSAFSVICDLGGASGALARVCASLYPDSSVLVLEVPE
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hsASMT 214 VVWTAKQHFSFQEEEQIDFQEGDFFKDPLPEADLYILARVLHDWADGKCSHLLERIYHTC
atASMT 233 VIEDAPSHP------GIEHVGGDMFV-SVPKGDAIFMKWICHDWSDEHCVKFLKNCYESL
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trASMT 202 VVRTSRENFFTEANQRIGFCEGDFFKDPLPEADLYVLARILHDWTDQRCLELLRRVHGAC
mamuASMT 214 VVRTAKQHFSFPEEEEIHLQEGDFFKDPLPEADLYILARILHDWADGKCSHLLERVYHTC
eeASMT 214 VVRAARSLFLSTVEAPVSFREGDFFKDPLPEADLYILARVLHDWTDEKCSELLAKIHHTC
consensus 241
hsASMT 274 KPGGGILVIESLLDEDRRGPLLTQ-LYSLN--MLVQ-TEGQERTPTHYHMLLSSAGFRDF
atASMT 286 PEDGKVILAECILPETPDSSLSTKQVVHVDCIMLAHNPGGKERTEKEFEALAKASGFKGI
btASMT 274 RTGGGILVIESLLDTDGRGPLTTL-LYSLN--MLVQ-TEGRERTPAEYRALLGPAGFRDV
trASMT 262 RPGGSVLLVEALLYEDGSGPLTAQ-LYSLN--MLVQ-TEGRERSAAQYAALLTAAGFSNV
mamuASMT 274 KPGGGILVIESLLDEDRRGPLLTQ-LYSLN--MLVQ-TEGQERTPTHYHMLLSSAGFRDF
eeASMT 274 KPGGGILVVESVLEEDRRGPLTTQ-LYSLN--MLVQ-TEGRERTPAEYRSLICSAGFQDF
consensus 301
hsASMT 330 QFK--KTGAIYDAILAR--K
atASMT 346 KVVCDAFGVNLIELLKK--L
btASMT 330 RCR--RTGGTYDAVLAR--K
trASMT 318 QHR--FTGKIYDAVLARKEA
mamuASMT 330 QFK--KTGAIYDAILVR--K
eeASMT 330 QLK--KTGKIYDVILAR--K
consensus 361

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/062513

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/10 C12P17/10
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/127915 A1 (UNIV DANMARKS TEKNISKE [DK]) 6 September 2013 (2013-09-06) cited in the application abstract; claim page 22 - page 23 page 27 - page 31 sequence 81 -----	1-4, 7-12,15
X	WO 2007/052166 A2 (PASTEUR INSTITUT [FR]; BOURGERON THOMAS [FR]; MELKE JONAS [SE]; GOUBRA) 10 May 2007 (2007-05-10) cited in the application figure 8 ----- -/--	1-4

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
23 June 2017	18/07/2017

Name and mailing address of the ISA/	Authorized officer
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